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Feline leukemia virus envelope protein expression encoded by a recombinant vaccinia virus: apparent lack of immunogenicity in vaccinated animals

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Summary

We have constructed a recombinant vaccinia virus encoding the expression of the feline leukemia virus (FeLV) envelope gene of the Gardner-Arnstein strain of FeLV subgroup B. Human cells infected with the recombinant virus (vFeLVenv) express and process the FeLV envelope protein similarly to cells infected with authentic FeLV. The mature gp70 protein is transported to and accumulates on the surface of vFeLVenv-infected cells. Vaccinia virus replication and FeLV gp70 accumulation was also observed in cells of feline and murine origin, albeit at levels somewhat reduced from those in human cells. Toward the goal of developing a recombinant vaccinia virus as a live vaccine for feline leukemia disease in cats, immunogenicity studies were performed in cats and mice. These experiments yielded surprising results: although animals mounted a typical virus-neutralizing antibody response to the vaccinia virus vector, we were unable to detect antibodies against FeLV gp70 in any of the vaccinated animals. A subsequent 'booster' immunization with killed FeLV was unable to elicit evidence of immunologic 'priming' by the recombinant virus. We are presently unable to explain the apparent lack of immunogenicity. These results may point to complexities involved in the development of vaccines to protect against retrovirus infection.

Recombinant vaccinia virus; Feline leukemia virus; Envelope gene expression; Retroviral vaccine

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Introduction

The feline leukemia viruses (FeLVs) comprise a group of horizontally transmitted type C retroviruses associated with a variety of naturally occurring diseases in domestic cats. FeLV infection causes proliferative and degenerative changes in cells of lymphoid and erythroid origin resulting in lymphosarcomas, leukemias, and anemias. Infected cats are often immunosuppressed and many succumb to secondary infections (Hardy et al., 1980).

Three subgroups of FeLV, A, B, and C, have been defined on the basis of viral interference (Sarma and Log, 1971). Subgroup A viruses represent the ecotropic members of this virus group. Viruses of subgroup B are believed to derive from in vivo recombination events between the ecotropic subgroup A virus and endogenous FeLV-like sequences within the cat genome (Stewart et al., 1986). This similarity with the mink cell focus-forming viruses of murine leukemia virus (MCF-MuLVs) had previously been suggested on the basis of amino acid sequence homology (Elder and Mullins, 1983). The subgroups differ in their natural distribution in that subgroup B and C viruses are found only in association with subgroup A virus (Jarrett et al., 1978). Specific differences with regard to infectivity and disease progression have also been noted (Jarrett and Russell, 1978).

As in other retrovirus systems, it is likely that many of these viral properties involve the products of the viral envelope (*env*) gene, specifically the major envelope glycoprotein gp70. The FeLV *env* gene encodes a precursor glycoprotein, gp85, which is proteolytically processed to yield the mature gp70 and p15E proteins. These proteins are complexed via disulfide linkages and are found on the membranes of infected cells and virions; the hydrophobic p15E protein is thought to anchor the complex in the lipid membrane. The major envelope glycoprotein gp70 is involved in host-receptor recognition and binding and is believed to serve as the primary target for antibody-mediated virus neutralization. We have previously defined a specific epitope of FeLV gp70 that is recognized by monoclonal antibodies that neutralize FeLV virus of all subgroups; synthetic peptides containing this epitope are themselves able to elicit virus-neutralizing antibodies in guinea pigs (Nunberg et al., 1984a, 1985).

Our efforts to develop a vaccine to protect cats from FeLV infection and disease have focused on the role of the FeLV *env* protein in immunoprophylaxis. One particularly attractive approach to immunization against FeLV disease involves the use of live, recombinant viruses that encode expression of the FeLV *env* gene in the course of vaccinal infection with a nonpathogenic virus vector. Vector systems based on the poxvirus, vaccinia virus, have been developed by several groups (Mackett et al., 1982; Panicali and Paoletti, 1982). The initial choice of vaccinia virus as a viral vector for vaccination derives from its historical role in smallpox vaccination (Behbehani, 1983) as well as from technical attributes of the system, including the ability of the virus to undergo in vivo marker rescue (Nakano et al., 1982; Weir et al., 1982). Recombinant vaccinia viruses encoding expression of a variety of heterologous vaccine immunogens have been constructed in several laboratories, and a number of these recombinant virus vaccines have been shown to elicit protective

immunity in animals upon vaccination (Panicali et al., 1983; Smith et al., 1983b; Moss et al., 1984; Paoletti et al., 1984; Wiktor et al., 1984; Cremer et al., 1985; Franke et al., 1985; Mackett et al., 1985). In this report we describe the construction of a recombinant vaccinia virus expressing the *env* gene of FeLV.

We were especially interested to develop a live virus vaccine for FeLV disease as a model to explore the immunobiology of retrovirus infection and immunoprophylaxis. Protective immunogens and protective immune responses to retrovirus infection have yet to be characterized. A recombinant vaccinia virus encoding the expression of the FeLV *env* protein provides a reagent with which to study the immunochemistry of this protein, as expressed within host cells and independent of the myriad effects of retrovirus infection. In this paper, we describe preliminary studies to assess the immunogenicity of the recombinant vaccinia virus as an initial step toward the development of an effective live virus vaccine to protect cats from FeLV infection and disease.

Materials and Methods

Cell lines and viruses

Human RD cells producing GA-FeLV virus (Mullins et al., 1981) were obtained from N. Davidson (Caltech). Canine CF-2 cells, expressing the molecularly cloned GA-FeLV provirus (Mullins et al., 1981), were provided by J. Elder (Research Institute of Scripps Clinic). The thymidine kinase-deficient human cell line, 143B (Weir et al., 1982) was from K. Huebner (The Wistar Institute) and murine L-929 cells were from L. Old (Memorial Sloan Kettering Cancer Institute). Human HeLa and feline CRFK (Crandell et al., 1973) cells were obtained from the American Type Culture Collection. The wild-type WR strain of vaccinia virus was obtained from B. Moss (NIH).

All cell lines, with the exception of CRFK, were grown in Dulbecco's Medium Eagle (DME; Irvine Scientific) supplemented with 10% fetal bovine serum (FBS) and gentamycin (50 µg/ml; Schering). CRFK cells were grown in McCoy's 5A (modified) medium (Gibco) supplemented as above.

Vaccinia virus was propagated by using standard techniques. Cell-associated vaccinia virus was isolated and purified by sucrose gradient centrifugation as described (Joklik, 1962); viral DNA was prepared from purified virions as described by Nakano et al. (1982). For quantitation or isolation of vaccinia virus plaques, inoculated cell monolayers (HeLa or 143B) were overlaid with medium containing 0.6% agarose, 4% FBS, and gentamycin at 50 µg/ml. The agarose overlay was additionally made 25 µg/ml in bromodeoxyuridine (BUdR) for the isolation of tk⁻ virus plaques. FeLV virus from persistently infected cells was partially purified from culture supernatants by using methods described by Frankel et al. (1976).

Specific reagents

The vaccinia virus insertion vectors pGS20 (Mackett et al., 1984) was provided by G. Smith and B. Moss (NIH). This plasmid serves as a vector to permit the insertion and expression of foreign genes within the genome of vaccinia virus.

The molecularly cloned GA-FeLV-B provirus (λ HF60; Mullins et al., 1981) was provided by A. Roach and N. Davidson (Caltech) in the form of the pKC7-derived plasmid pKHR-1. This plasmid is infectious in transfection assays. The plasmid was pared down, to contain only the FeLV *env* gene and 3' LTR, by intramolecular recircularization following digestion with *Pvu*II. This smaller plasmid, pGApv, provided the FeLV *env* gene sequence used in these studies.

The FeLV gp70-specific monoclonal antibody, clone 25.5, has been previously described (Lutz et al., 1981). This IgG1 antibody neutralizes FeLV infectivity and recognizes a defined determinant within FeLV gp70 (Nunberg et al., 1984a). Horseradish peroxidase (HRP) conjugates of the antibody, for enzyme-linked immunosorbent assays (ELISA), were prepared as described (Lutz et al., 1983). Another virus-neutralizing monoclonal antibody that recognizes the same epitope (C11D8; unpublished) was provided by C. Grant (Pacific Northwest Research Foundation; Grant et al., 1983); this IgG2 antibody was used in experiments requiring antibody binding to *Staphylococcus* protein A.

Recombinant DNA methods

The recombinant DNA procedures used were as described by Maniatis et al. (1982). Nucleic acid enzymes were obtained from New England Biolabs or Bethesda Research Laboratories and were used as described by the manufacturer.

Recombinant vaccinia virus methods

The general procedures used to derive recombinant vaccinia viruses have been described (Mackett et al., 1984). Vaccinia virus-infected 143B cells were transfected with vaccinia virus DNA and the pGS20-derived insertion vector containing the FeLV *env* gene (pVGA), and progeny virus were grown in the presence of BUdR (25 μ g/ml) to select for recombinant (tk^-) genomes. BUdR-resistant viruses were plaque-purified and screened for insertion and expression of the FeLV *env* gene as follows: monolayers of HeLa cells were infected with candidate viral isolates and harvested 2 days later by lysis in 0.1 M Na_2CO_3 , pH 9 (ELISA coating buffer). A portion of the lysate was used directly in an ELISA assay to detect FeLV gp70. The lysate was used to coat microtiter wells, and FeLV *env* protein was detected using HRP-conjugated monoclonal antibody 25.5 and 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS; Sigma) substrate (Lutz et al., 1981). The remainder of the lysate was used to isolate high molecular weight DNA for dot hybridization analysis. The lysate was made to 0.05 M Tris (pH 7.0), 0.01 M EDTA, 0.5% SDS and was treated with proteinase K (50 μ g/ml; Sigma) as described by Gross-Bellard et al. (1973). Following extraction with phenol and precipitation with ethanol, the DNA was redissolved in 0.1 M NaOH, 0.05 M EDTA for dot hybridization analysis as described by Kafatos et al. (1979).

Immunological analysis of FeLV env protein expression

Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). In some experiments, crude preparations of membrane proteins were isolated from virus-infected cells: cells were swollen in 9 volumes cold hypotonic buffer (0.01

M Tris, pH 7.4, 0.01 M NaCl, 0.001 M MgCl_2) and disrupted by Dounce homogenization. Nuclei were removed by low-speed centrifugation ($1000 \times g$, 10 min) and the membrane fraction was subsequently isolated by high-speed centrifugation at $29000 \times g$ for 60 min. Protein content was determined by the method of Lowry et al. (1951).

Immunoblot analysis was performed on proteins resolved by polyacrylamide gel electrophoresis and transferred electrophoretically to nitrocellulose (BA-85; Schleicher and Schuell) (Towbin et al., 1979; Burnette, 1981). Blots were treated and incubated with antibody as previously described (Johnson et al., 1984; Nunberg et al., 1984a), and antibody-antigen complex was visualized using a HRP-conjugated second antibody (rabbit anti-mouse; Accurate Chemicals) and 3,3'-diaminobenzidine (Sigma) as the enzyme substrate (De Blas and Cherwinski, 1983).

Live cell indirect immunofluorescence experiments (Manger et al., 1984) were performed to localize FeLV *env* protein on the surface of vaccinia virus infected cells. Infected cell monolayers, grown in tissue culture chamber slides (Lab-Tek), were washed gently with phosphate-buffered saline (PBS) prior to incubation with monoclonal antibody 25.5 in PBS containing 4% bovine serum albumin (BSA). Following additional careful washing, immune complexes were visualized by reaction with a fluorescein-conjugated second antibody (rabbit anti-mouse IgG; Cappel Laboratories). Parallel monolayers were fixed in cold (-20°C) acetone prior to incubation with 25.5 antibody in order to detect total cellular FeLV *env* antigen. Slides were examined and photographed by ultraviolet epifluorescence microscopy.

Immunoprecipitation experiments were performed to further differentiate between FeLV *env* protein localized intracellularly or on the surface of infected cells. Infected cells were metabolically labelled during 19 h of growth in methionine-free DME medium containing [^{35}S]methionine ($63 \mu\text{Ci/ml}$; New England Nuclear). In vaccinia virus infection, cells were labelled following a 2 h inoculation period. The intact monolayers were washed with PBS and incubated with C11D8 monoclonal antibody (in PBS containing 1 mg/ml of BSA) in order to obtain reaction with FeLV *env* antigen on the surface of cells. Monolayers were then extensively washed prior to lysis with RIPA buffer containing 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.01 M methionine, 0.005 M EDTA, 0.005 M 2-mercaptoethanol, 1 mg/ml BSA, and 100 U/ml aprotinin (Boehringer-Mannheim) in PBS. Immune-complexes were precipitated using *Staphylococcus* protein A-Sepharose (Sigma) and washed. Parallel immunoprecipitations were performed from cultures that had been lysed prior to incubation with antibody. Immunoprecipitated proteins were resolved by polyacrylamide gel electrophoresis and visualized by fluorography.

Animal immunogenicity studies

Animal studies were carried out in isolation facilities, and in accordance with recommendations of the National Institutes of Health. Female 6–8-week-old Balb/c mice were purchased from Charles River Laboratories, Inc. (Wilmington, Mass.). Specific (FeLV) pathogen-free cats, 12 weeks of age and of random sex, were obtained from the breeding colonies of the Feline Leukemia Research Laboratory, Univ. California, Davis. In some cases, animals were immunized with formalin-killed

FeLV virus (Pedersen et al., 1986) in incomplete Freund's adjuvant to attempt to 'boost' an immune response to the FeLV *env* protein (Nunberg et al., 1985).

Antibody to FeLV gp70 protein was determined in an ELISA assay as described by Pedersen et al. (1986). Purified FeLV gp70 protein used as antigen in the ELISA was prepared by immunoaffinity chromatography (Pedersen et al., 1986). Additional ELISA assays were performed using as antigen membrane preparations from FL-74 lymphosarcoma cells producing FeLV-A, B, and C (Pedersen et al., 1986).

Results and Discussion

Derivation of recombinant vaccinia virus containing FeLV env gene

The general procedures used to construct the recombinant vaccinia virus containing the FeLV *env* gene were developed by B. Moss and his colleagues (Mackett et al., 1984) and have been widely applied to generate a variety of recombinant vaccinia viruses expressing foreign genes (Smith et al., 1983a,b, 1984; Wiktor et al., 1984; Cremer et al., 1985; Franke et al., 1985; Mackett et al., 1985; Rice et al., 1985). The plasmid vector utilized to mediate homologous recombination and insertion of the FeLV *env* gene into the vaccinia virus genome was derived from the previously described plasmid pGS20 (Mackett et al., 1984). The pGS20 plasmid contains the vaccinia virus thymidine kinase (*tk*) gene interrupted by the promoter region of the vaccinia virus gene encoding an early '7.5 kDa' protein (Venkatesan et al., 1981). A polylinker sequence has been inserted downstream of the transcription initiation site of the '7.5 kDa' gene in order to facilitate molecular cloning of heterologous sequences at this location.

The FeLV *env* gene was obtained from an infectious proviral isolate of the Gardner-Arnstein strain of FeLV (subgroup B) (GA-FeLV-B) (Mullins et al., 1981). A 3.0 kb *Pst*I-*Eco*RI DNA fragment containing the FeLV *env* gene was inserted within the polylinker of pGS20 so as to ensure the proper relative orientation of vaccinia '7.5 kDa' promoter and FeLV *env* gene (Fig. 1). The fragment contains the entire *env* gene and FeLV LTR and terminates at an *Eco*RI site within human flanking sequences approximately 0.4 kb downstream of the 3' end of the integrated provirus (Elder and Mullins, 1983; Nunberg et al., 1984b). The translation initiation codon of the *env* gene is located 59 nucleotides from the *Pst*I site at the 5' end of the inserted fragment. As constructed, there are no transcribed AUG codons preceding that which initiates translation of authentic *env* gene product (unpublished).

The resulting pGS20-derived plasmid, pVGA (Fig. 1), was used to derive the recombinant vaccinia virus (Mackett et al., 1984); the plasmid was introduced into vaccinia virus-infected cells to permit homologous recombination between plasmid and vaccinia genomic *tk* sequences. Progeny virus were collected and subjected to selection in *tk*⁻ 143B cells in the presence of BUdR. Under these conditions, only virus rendered defective in *tk*, by virtue of insertion of the pVGA cassette within the viral *tk* gene or by spontaneous mutation, will replicate.

Recombinant viruses containing the FeLV *env* gene were identified among the

CONSTRUCTION OF FELV_{env} GENE INSERTION VECTOR

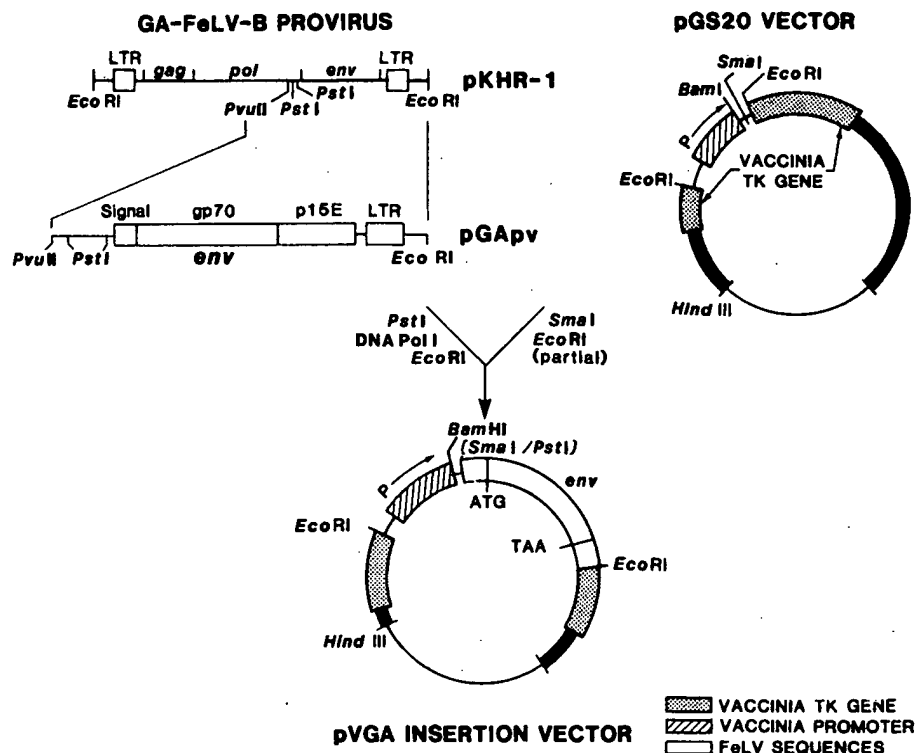


Fig. 1. Construction of pVGA: a pGS20-derived plasmid containing the FeLV envelope gene. The construction of the insertion vector pVGA is outlined. The final pVGA plasmid was identified by colony hybridization to ampicillin-resistant transformants in *E. coli* mm294, using a nick-translated FeLV *env*-specific probe, followed by restriction endonuclease digestion analysis. The drawings are not to scale, and some *Pst*I sites within the pKHR-1 provirus are not indicated. Restriction endonuclease sites included within brackets are not regenerated during plasmid construction.

spontaneous tk⁻ viruses by using two rapid screening procedures: (i) hybridization analysis of infected HeLa cells to identify virus containing FeLV sequences, and (ii) immunological analysis of infected cells to identify virus encoding the expression of FeLV gp70 protein. Virus was isolated from well-separated plaques after BUdR-selection and used to infect cells in microtiter dishes. Infected cell monolayers were harvested 2 days later and processed so as to provide DNA for dot hybridization analysis and protein for ELISA analysis. No effort was made to control for multiplicity or extent of infection. Although these assays are relatively crude and qualitative, results were generally concordant and multiple isolates were obtained that contain and express the FeLV *env* gene. These virus stocks were further plaque-purified and one isolate (vFeLV_{env} 2165) was chosen for further analysis.

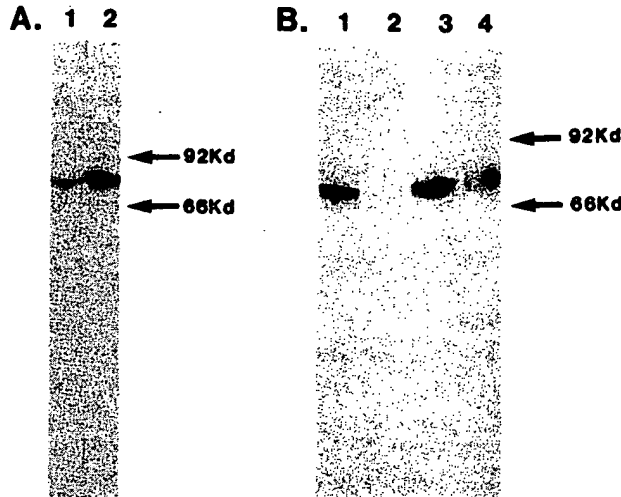


Fig. 2. Immunoblot analysis of vFeLVenv-encoded expression of FeLV *env* gene. Proteins were prepared from HeLa cell monolayers infected with the recombinant vaccinia virus vFeLVenv, or with the wild-type WR vaccinia virus, and expression of FeLV *env* protein was determined by immunoblot analysis using the FeLV gp70-specific 25.5 monoclonal antibody. The procedures are referenced in Materials and Methods. Lanes are as follows: Panel A: (1) total cell protein (200 μ g) from HeLa cells infected with vFeLVenv, (2) cell membrane protein (200 μ g) from parallel culture of HeLa cells infected with vFeLVenv; Panel B: (1) cell membrane protein (150 μ g) from HeLa cells infected with vFeLVenv, (2) cell membrane protein (250 μ g) from HeLa cells infected with WR vaccinia virus, (3) cell membrane protein (250 μ g) from canine CF/2 cells expressing authentic molecularly cloned GA-FeLV-B virus, and (4) partially purified GA-FeLV virus from culture supernatants of CF/2 cells, above.

Expression of the FeLV env gene in vFeLVenv-infected cells

Expression of the FeLV *env* gene in HeLa cells infected with the recombinant vFeLVenv virus was examined by using the 25.5 monoclonal antibody directed against FeLV gp70. Immunoblot analysis of proteins expressed by vFeLVenv-infected HeLa cells revealed a single band of immunoreactive material that migrated with an apparent molecular size of 83kD (Fig. 2A, lane 1). This protein was enriched in membranes prepared from these cells (lane 2); this is in keeping with the expected membrane association of the *env* gene product. A protein of identical molecular size was observed in membrane preparations of dog cells productively infected with authentic GA-FeLV virus (Fig. 2B, compare lanes 1 and 3).

The apparent molecular size of the 83 kD protein does not in itself distinguish between the two potential forms of the FeLV *env* gene product—the 'gp85' precursor protein or the processed, mature 'gp70' protein. To obtain a molecular size marker for mature 'gp70' of GA-FeLV, FeLV virions containing 'gp70' were partially purified from culture supernatants of GA-FeLV-infected CF/2 canine cells. Immunoblot analysis of virion protein (Fig. 2B, lane 4) revealed a band that comigrated with those previously observed in membrane preparations of cells infected with either vFeLVenv or authentic GA-FeLV. Thus, the *env* gene product

that accumulates in these cells is the mature, processed form of the protein, gp70. (This assignment has been confirmed by molecular size analysis of the protein product after treatment with anhydrous hydrogen fluoride (Mort and Lamport, 1977) to remove carbohydrate moieties (51 kDa; data not shown).) The apparent 83 kDa molecular size of the GA-FeLV 'gp70' glycoprotein likely reflects the large number of potential glycosylation sites present within this protein relative to other retroviral 'gp70' proteins (Elder and Mullins, 1983).

In these analyses of steady-state protein levels, we see no accumulation of a higher molecular size 'gp85' precursor protein. This is consistent with the short half-life measured for the gp85 precursor protein in the MuLV system (Famulari et al., 1976). Although we have not specifically measured FeLV p15E accumulation, we presume that proteolytic processing of the gp85 precursor molecule occurs to generate p15E protein coordinately with the observed gp70. That proteolytic processing to generate mature gp70 occurs efficiently in cells infected with vFeLVenv demonstrates that cleavage of the gp85 precursor does not require the presence of other FeLV-encoded proteins and is independent of the process of FeLV virus assembly and budding.

The *env* gene product in FeLV-infected cells is processed and transported to the cell surface membrane, from which it is shed as budding viral particles. To determine the subcellular localization of the vFeLVenv-encoded FeLV gp70 protein, indirect immunofluorescence studies were performed using the 25.5 monoclonal antibody to stain the surface of intact cells (Fig. 3). In cells permeabilized by treatment with cold acetone, intracellular *env* protein could be detected as early as 3–6 h post-infection. Surface fluorescence in intact cells was visible after 6 h; by 24 h an intense staining pattern typical of surface expression was observed in vFeLVenv-infected cells. Thus, the *env* gp70 protein is transported to, and accumulates on, the surface of cells infected with the recombinant vaccinia virus.

To quantitate these findings, and to compare the transport of the *env* gene product in vFeLVenv-infected cells with that in cells infected with FeLV, immunoprecipitation experiments were performed so as to distinguish between intracellular and surface expression of gp70 antigen (see Materials and Methods). Results from these studies, comparing cell surface and total expression of FeLV gp70 in cells infected with the recombinant vaccinia virus vFeLVenv and those persistently infected with authentic GA-FeLV, are presented in Fig. 4 (lanes 3 and 4, respectively; note the difference in cell numbers used in panels). The gp70 protein in vFeLVenv-infected cells appears to partition roughly equally between intracellular and cell-surface membranes. In cells productively infected with FeLV, less than 10% of the gp70 protein accumulates on the cell surface; this presumably reflects the continual budding of gp70-containing virions from the surface of the FeLV-infected cells. Although virus budding makes it impossible to relate gp70 accumulation and synthesis in this experiment, it is apparent from these results that cells infected with the recombinant vaccinia virus accumulate substantially more gp70 than do cells infected with FeLV.

In summary, we find no qualitative differences in the synthesis, processing, and transport of the FeLV *env* protein when expressed in cells infected with the

vFeLV *env* : FIXED-CELL STAINING**vFeLV *env* : SURFACE STAINING**

Fig. 3. Indirect immunofluorescent staining of vFeLV env -infected cells. HeLa cell monolayers were infected with the recombinant vaccinia virus vFeLV env at a low multiplicity of infection and examined by indirect immunofluorescence 24 h after infection. FeLV env protein was detected using the FeLV gp70-specific monoclonal antibody 25.5 as described in Materials and Methods. Panel A shows whole cell immunofluorescence in cells fixed and permeabilized in cold acetone; Panel B shows live-cell immunofluorescence in which only FeLV env protein expressed on the surface of infected cells is detected. Exposure times for the photomicrographs were 0.5 min and 2 min, respectively.

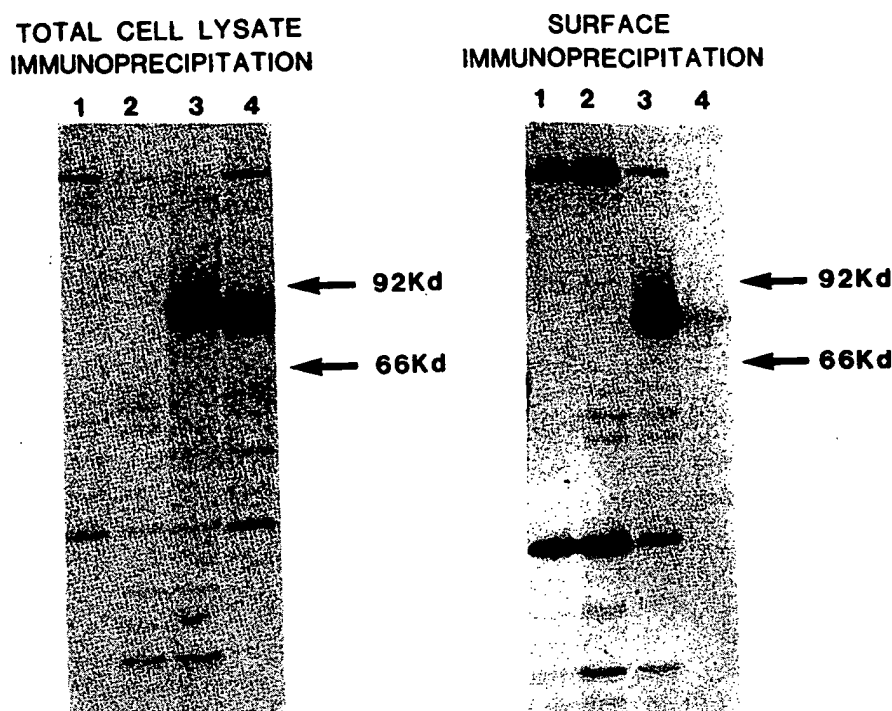


Fig. 4. Immunoprecipitation of cell-surface FeLV *env* protein from vFeLVenv-infected cells. HeLa cell monolayers were infected with vaccinia virus and metabolically labelled with [35 S]methionine for 19 h. Human RD cells persistently infected with authentic GA-FeLV were also labelled for 19 h. The intact monolayers were then incubated with the FeLV gp70-specific C11D8 monoclonal antibody and processed as described in Materials and Methods so as to obtain immunoprecipitation of FeLV *env* protein exposed on the cell surface ('surface immunoprecipitation' from 1×10^7 cells). Parallel cultures were lysed prior to incubation with antibody ('total cell lysate immunoprecipitation' from 2.5×10^6 cells). Proteins were resolved by SDS-gel electrophoresis and visualized by fluorography. Lanes are as follows: (1) uninfected HeLa cells, (2) HeLa cells infected with WR virus, (3) HeLa cells infected with vFeLVenv virus, and (4) human RD cells infected with GA-FeLV.

recombinant vaccinia virus, vFeLVenv, or in cells infected with FeLV. Proteolytic processing of the *env* gp85 precursor, to yield mature gp70 and, presumably, p15E, occurs rapidly during transit to the cell surface. In the absence of FeLV virion budding, gp70 protein accumulates on the surface of vFeLVenv-infected cells.

Expression of vFeLVenv-encoded FeLV env gene in feline cells

Toward our goal of developing a recombinant vaccinia virus as a live vaccine for feline leukemia disease, we were interested to examine vaccinia virus replication and FeLV *env* gene expression in cells of feline origin. From the veterinary literature (Gaskell et al., 1983; Martland et al., 1983), and from our own safety studies (unpublished), it was known that vaccinia virus is able to replicate in cats. To

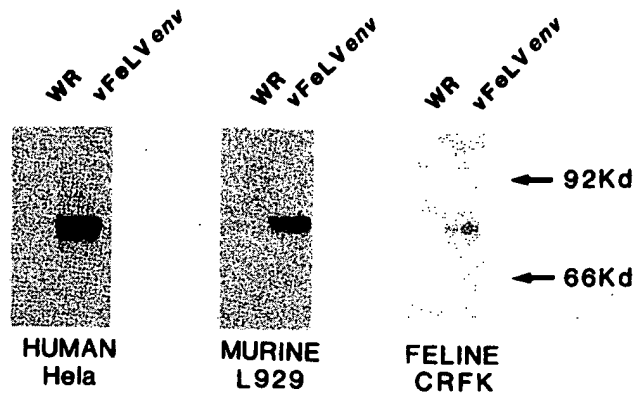


Fig. 5. vFeLVenv-encoded *env* protein expression in human, murine, and feline cells. Comparable cell monolayers of human HeLa cells, murine L929 cells and feline CRFK cells were infected with recombinant vFeLVenv virus, or with WR vaccinia virus. In all cases infection was at a multiplicity of infection approximately equal to one, as determined by plaque assay on HeLa cells. Cells were harvested 2 days post-infection and membrane preparations (100 μ g protein) were analyzed to determine FeLV *env* protein. Immunoblot analysis was using the FeLV gp70-specific 25.5 monoclonal antibody.

quantitate these aspects, cells of feline, murine, and human origin (CRFK or Fc9, L929, and HeLa or 143B, respectively) were infected with the recombinant vaccinia virus, and virus production and FeLV *env* protein accumulation were measured. Comparable cell monolayers were infected at a multiplicity of infection of one and cells were harvested at 48 h post-infection. Cell-associated virus was quantitated in a plaque assay using HeLa cells, and gp70 accumulation was determined immunologically from membrane preparations of infected cells.

These experiments confirmed that substantial vaccinia virus replication occurs in cells of feline origin. Virus yields obtained in feline cells were, however, substantially reduced from those obtained in human cells (10^8 – 10^9 PFU vs. 10^{10} – 10^{12} PFU per 10^7 cells, respectively). Virus replication in feline cells was further evidenced by the extensive, albeit slower, cytopathic effect observed in infected feline cell monolayers. A similar pattern of virus growth was observed in cells of murine origin; virus yields obtained in mouse L929 cells were generally comparable to those obtained in cat cells.

FeLV gp70 protein expression in these cell lines (Fig. 5) appeared to correspond to virus production. That is, accumulation of gp70 protein in feline and murine cells was substantially reduced as compared to that in human cells. (Although virus yields were comparable in infected murine and feline cells, murine cells were consistently found to accumulate higher levels of FeLV gp70. The basis of this difference is unknown.) In all cell lines examined, virus yields were similar in cells infected with the wild-type WR virus or with the recombinant vFeLVenv virus (data not shown). Expression of the FeLV *env* gene product did not appear to affect the level of virus production.

Immunogenicity of vFeLVenv-encoded FeLV gp70 in cats and mice

Studies were performed to assess the immunogenicity of the FeLV gp70 protein produced upon vaccination of cats and mice with the live, recombinant virus. Female Balb/c mice (5 per group) were inoculated with approximately 2×10^9 PFU of purified vFeLVenv by either intradermal or subcutaneous routes; control groups were injected intradermally with 10^9 PFU of WR vaccinia or with buffer. All animals were re-inoculated at 2 weeks. FeLV-free kittens, 12 weeks of age, were used in immunogenicity studies in cats; two kittens received the recombinant vFeLVenv virus (10^9 PFU) and one received the wild-type WR virus (10^9 PFU). Animals were vaccinated on the ventral abdomen, by the intradermal route, at weeks 0, 4, and 8.

In both species, typical vaccinia virus lesions formed locally at the site of primary vaccination with either wild-type or recombinant virus. These lesions consisted of raised, erythematous plaques 2–5 mm in diameter that appeared by the 3rd through 5th day. The lesions reached maximal size by day 7–9 and then rapidly regressed, often leaving small scabs. Lesions generated by the recombinant virus appeared somewhat less severe than those of the WR virus; both were distinctly less fulminating than typically seen in primary vaccination in humans. No dermal response to secondary vaccination was noted, indicating an effective immune response to the primary vaccinia virus infection. Serum samples were obtained throughout the vaccination protocol; antibody to the vaccinia virus vector was determined by virus-neutralization and antibody to FeLV gp70 was determined using an established ELISA assay (Nunberg et al., 1985; Pedersen et al., 1986). All vaccinated animals mounted an immune response to the vaccinia virus. Vaccinia virus neutralizing antibody titers of 1:25 to 1:125 were detected in all vaccinated animals, consistent with titers reported by others (Wiktor et al., 1984; Franke et al., 1985). The surprising result obtained from these studies was that we were unable to detect anti-gp70 antibody in either species at any time following primary or secondary vaccination with the recombinant vaccinia virus. The specific lack of antibody to the vFeLVenv-encoded FeLV gp70 protein was truly unexpected.

We were concerned that the expression of the FeLV *env* gene might be unstable during *in vivo* infection. To examine the stability of the recombinant virus, biopsies of primary lesions were obtained from kittens 5 days following vaccination with the recombinant virus. Viruses obtained from these skin biopsies were still able to encode the expression of FeLV gp70, as determined by immunostaining of viral plaques (data not shown). Thus, expression of the FeLV *env* gene appeared to be stably maintained throughout *in vivo* infection.

To obtain another, perhaps more sensitive, indication of an immune response to FeLV gp70 produced on vFeLVenv infection, we were interested to determine whether vaccination had 'primed' an immune response to gp70. Emini et al. (1983) had previously described a poliovirus VP1-derived synthetic peptide that, although in itself unable to elicit a humoral response, was able to 'prime' a response that could be manifested through a subsequent 'boost' with a subthreshold dose of live poliovirus. We have described a similar phenomenon in the response of guinea pigs to a synthetic peptide containing a region of FeLV gp70 protein that is recognized

by virus-neutralizing monoclonal antibodies (Nunberg et al., 1985); in this case, subsequent immunization with killed FeLV virus was able to 'boost' a virus-neutralizing antibody response. To test if vaccination with the recombinant vaccinia virus was able to 'prime' a humoral response to FeLV gp70, vaccinated mice and cats were immunized with formalin-killed FeLV after completion of the vaccination protocol. The single injection of killed FeLV used in these experiments does not by itself elicit anti-gp70 antibody in control animals of either species. Nor, in these studies, did it 'boost' anti-gp70 antibody in animals that had previously been vaccinated with the recombinant vaccinia virus. Thus, by this assay, we could not detect immunologic 'priming' of a response to vFeLVenv-encoded gp70 protein.

Moss et al. (1984) have previously reported the absence of a detectable humoral immune response in chimpanzees vaccinated with a recombinant vaccinia virus encoding expression of the hepatitis B surface protein. In these studies, vaccinated animals did manifest a 'primed' immune response upon challenge and were protected. Whether cats vaccinated with the recombinant vFeLVenv virus in this study would 'boost' upon FeLV challenge, or whether protection would be afforded via other immune mechanisms, is unknown. In the absence of an antibody response, these cats were not challenged with virulent FeLV.

Several hypotheses can be advanced to attempt to explain the apparent lack of immunogenicity of the vFeLVenv-encoded gp70 in cats. These might, for instance, postulate the establishment of immune-tolerance to the *env* protein of the MCF-like FeLV-B used in these studies, as a result of the possible expression of endogenous FeLV-like sequences during feline development. Or, one might attribute the apparent lack of immunogenicity to the reported immuno-suppressive activity of the p15E protein of FeLV (Hebebrand et al., 1977; Mathes et al., 1978). Alternatively, various hypotheses might spring from the paucity of definitive information regarding the immunochemistry of the FeLV *env* protein. Although FeLV gp70 protein is immunogenic in cats on infection (Lutz et al., 1980; Pedersen et al., 1985) and immunization (Salerno et al., 1978; Pedersen et al., 1986), it is reported to be so only weakly. Paradoxically, although passive protection from FeLV infection can be afforded by using goat antibodies to the homologous gp70 protein of Friend-MuLV (deNoronha et al., 1978), attempts to elicit protection in cats with purified FeLV gp70 protein have to date been unsuccessful (Pedersen et al., 1986). Nonetheless, protective humoral immunity can be generated in cats in natural infection (Hoover et al., 1977; Pedersen et al., 1977) and, in some studies via immunization with killed FeLV (Hoover et al., 1977; Pedersen et al., 1979, 1986; Haley et al., 1985; but see also Olsen et al., 1977; Schaller et al., 1977). Limited protection has also been claimed using a recently approved commercial vaccine (LeukocellTM, Norden Laboratories, Lincoln, NE) consisting of inactivated culture supernatants containing protein products from FeLV-transformed cells (Lewis et al., 1981). In general, however, current immunization procedures, using commercial or experimental vaccines, do not elicit strong, universal protection.

It is more difficult to construct hypotheses regarding the apparent lack of immunogenicity of the vFeLVenv-encoded gp70 in mice. Although vaccinia virus replication and resultant FeLV *env* protein expression is somewhat restricted in cells

of feline and murine origin, this has not in and of itself limited the immunogenicity of other recombinant vaccinia viruses in mice (Bennink et al., 1984; Paoletti et al., 1984; Wiktor et al., 1984; Mackett et al., 1985; Cremer et al., 1985). Other hypotheses, amenable to experimental study in the mouse but not the cat, relate to the role of the major histocompatibility complex in defining the immune response. The immunogenetics of retrovirus infection are quite complex, and a large number of H2-linked and H2-independent genetic loci have been determined to affect the outcome of infection in the mouse (Merulo and Bach, 1983). In vaccination with a recombinant vaccinia virus, one might also assume that the vaccinia virus is not simply a passive vector and that histocompatibility complex restrictions may independently apply in the immune response to heterologous immunogens when expressed during vaccinal infection, or to vaccinal infection per se (Earl et al., 1986).

None of the hypotheses for the apparent lack of immunogenicity of the vFeL-Venv-encoded FeLV *env* protein is entirely satisfactory or compelling. At present, we cannot explain the unexpected lack of humoral immune response to the FeLV gp70 protein.

Much remains to be understood regarding the immunochemistry of retrovirus *env* gene products and the role of these proteins in retrovirus infection and immunoprophylaxis. Recombinant vaccinia viruses expressing retrovirus *env* gene products provide a means to study the immunobiology and function of the proteins independent of the myriad effects of retrovirus infection. The surprising lack of immunogenicity seen with the present recombinant virus may point to complexities involved in the development of vaccines against retrovirus infection.

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